REMARKS

Claims 40-65 are pending in this application. Claims 1-39 have been cancelled without prejudice or disclaimer. Claims 45-46 and 51-65 have been withdrawn as being directed to non-elected subject matter.

Claims 1-39 have been canceled without prejudice or disclaimer, for the sole reason of advancing prosecution. Applicant reserves the right to reassert any of the claims canceled in a continuing application.

Independent claim 40 is directed to a "method for diagnosing breast cancer in a subject comprising determining levels of expression of p14 peptide in one or more samples from said subject, a high level of expression signifying a high probability for breast cancer in said subject." Claims 41-44 depend, either directly or indirectly from claim 40.

Independent claim 47 is directed to a "method for screening samples into such which signify that subjects from which they were obtained have a relatively high possibility of having or being susceptible of developing breast cancer and such which signify that subjects from which they were obtained have a relatively lower probability of having or being susceptible of developing breast cancer, the method comprising contacting the samples with anti-p14 antibodies and determining binding of anti-p14 antibodies and p14 peptide in said sample, a high degree of binding signifying a corresponding higher probability of having or being susceptible of developing breast cancer." Claims 48-50 depend, either directly or indirectly from claim 47.

In view of the remarks set forth below, further and favorable consideration is respectfully requested.

I. At page 2 of the Official Action, claims 40-44 and 47-50 have been rejected under 35 USC § 112, second paragraph.

The Examiner asserts that claims 40 and 47 are indefinite for reciting either "high level of expression" or "high degree of binding." Further the Examiner asserts that claims 42 and 48 are indefinite because the phrase suspicious area of the breast" is allegedly subjective.

In view of the following, this rejection is respectfully traversed.

With regard to the phrase "high level of expression," Applicant notes that the present specification, at paragraph 28 of the published present application, provides that:

> "High level of expression" denotes a level significantly (e.g. as determined by statistical determination) higher than a standard. "Standard" as used herein denotes either a single standard value or a plurality of standards with which the level of expression p14 peptide from the tested sample is compared. The standards may be provided, for example, in the form of discrete numeric values or is calorimetric in the form of a chart with different colors or shadings for different levels of expression; or they may be provided in the form of a comparative curve prepared on the basis of such standards. ....

The Examiner asserts that it is not clear how samples from breast cancer patients may be used as standards. However, Applicant submits that, as known to those of ordinary skill in the art, it should be appreciated that standards are typically determined once, and then can be used for future examinations. In the present application, page 6 provides that:

> ...the standards may be prepared by determining the level of expression p14 peptide present in a sample obtained from a plurality of patients positively diagnosed (by other means, for example by a physician, by histological techniques etc.) as having breast cancer at varying levels of severity (being correlated with level of expression of p14).

In other words, a set of cancer patients is used to determine the standards, and then these standards are used for future diagnosis of breast cancer in other subjects susceptible of having breast cancer.

Similarly, the term "high degree of binding" should be understood as a binding higher than a predetermined standard, for example, signifying a healthy state. In this regard, Applicant notes that although the specification does not provide an express definition for this phrase. The meaning of "high degree of binding" high degree of binding would be clear to a person of ordinary skill in the art.

With regard to the phrase "suspicious area in the breast," Applicant submits that a skilled artisan reading, for example page 4 of the present specification would able to ascertain the full meaning of this phrase. In this regard, page 4 of the specification provides that:

> The biopsy specimen includes any sample (tissue or liquid) obtained from a suspicious area in the breast, such as from a breast lump, of a subject suspected of having breast cancer.

Taken together with the level of skill in the art, a skilled artisan would understand the phrase "suspicious area in the breast" to include an area of the breast, which is observed as being abnormal such as, for example, a breast lump.

In view of the foregoing, it is submitted that claims 40-44 and 47-50 are clear and definite within the meaning of 35 USC § 112, second paragraph. Therefore, the Examiner is respectfully requested to withdraw this rejection.

II. At pages 3-5 of the Official Action, claims 40-44 and 47-50 have been rejected under 35 USC § 103(a) as unpatentable over Pogo (US Patent No. 6,040,146) in view of Hoch-Marchaim 2003 (of record), in further view of Melana I (of record) and Melana II (of record).

The Examiner asserts that it would have been obvious to combined the teachings of Pogo with Hoch-Marchaim, Melana I and Melana II to make the claimed method for diagnosing breast cancer.

In view of the foregoing, Applicant respectfully traverses the rejection of claims 40-43 and 47-49.

To establish a *prima facie* case of obviousness, the PTO must satisfy three requirements. First, as the U.S. Supreme Court very recently held in *KSR International Co. v. Teleflex Inc. et al.*, 550 U. S. 398 (2007), "a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions. ...it [may] be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art; all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. ...it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does... because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known." (*KSR*, 550 U.S. at 417). Second, the proposed modification of the prior art must have had a reasonable expectation of success,

determined from the vantage point of the skilled artisan at the time the invention was made. *Amgen Inc. v. Chugai Pharm. Co.*, 18 USPQ2d 1016, 1023 (Fed. Cir. 1991). Lastly, the prior art references must teach or suggest all the limitations of the claims. *In re Wilson*, 165 USPQ 494, 496 (C.C.P.A. 1970).

Regarding motivation to modify properly combined references, **MPEP 2143** states that where the prior art conflicts, all teachings must be considered and that the fact that references can be combined or modified is not sufficient to establish *prima facie* obviousness. **MPEP 2143** further states that there must be some suggestion or motivation to modify the references, and there must be a reasonable expectation of success. In addition, the prior art reference or references when properly combined, must teach or suggest all the claim limitations.

MPEP 2143.01 states that a proposed modification cannot render the prior art unsatisfactory for its intended purpose. If it does, then there is no suggestion or motivation to make the proposed modification. Further, the proposed modification cannot change the principle operation of a reference.

It is submitted that a proper case of *prima facie* obviousness has not been established because there would not be a reasonable expectation of success that the cited references could be modified to arrive at the presently claimed subject matter.

Independent claim 40 is directed to a "method for diagnosing breast cancer in a subject comprising determining levels of expression of p14 peptide in one or more samples from said subject, a high level of expression signifying a high probability for breast cancer in said subject." Claims 41-43 depend, either directly or indirectly from claim 40.

Independent claim 47 is directed to a "method for screening samples into such which signify that subjects from which they were obtained have a relatively high possibility of having or being susceptible of developing breast cancer and such which signify that subjects from which they were obtained have a relatively lower probability of having or being susceptible of developing breast cancer, the method comprising contacting the samples with anti-p14 antibodies and determining binding of anti-p14 antibodies and p14 peptide in said sample, a high degree of binding signifying a corresponding higher probability of having or being susceptible of developing breast cancer." Claims 48-49 depend, either directly or indirectly from claim 47.

Applicant respectfully submits that as evidenced by, and taken in combination with, Hoch-Marchaiam et al., *Localization of Mouse Mammary Tumor Virus Protiens in T-Cell Lymphoma,* Virology 242:245-254 (1998) (hereinafter "***Hoch-Marchaim 1998***", enclosed herewith as Attachment A), there would be no reasonable expectation of success that the cited references could be modified to arrive at the presently claimed subject matter. Specifically, Applicant submits, in various cell lines acceptable as models of breast cancer, the presence of p14 was not detected to be used as the marker for diagnosing human breast cancer in accordance with the presently claimed subject matter.

In this regard, Applicant notes in Hoch-Marchaim 1998, the present inventor described that p14, i.e., the leader peptide of the MMTV env-precursor protein, is localized to the nucleoli of T-cell lymphomas that harbor the virus. Applicant submits that was the first time that such a finding was made despite many years of MMTV

research. Hoch-Marchaim 1998 demonstrates that MMTV-positive cells of non-lymphocytic origin are devoid of both p14 and p21. See Abstract).

Further, Hoch-Marchaim 1998 report in the *Results* section that based on Western Blot analysis with M-66, either the p14 or p21 protein were devoid from MMTV-negative PIR-2 and 136.5 murine T-cell lymphomas, MMTV-positive mammary carcinoma cell line Mm5MT, two independent CZECH 2 MMTV-positive mammary tumor tissues, and Syngeneic Balb/C spleen and liver cells.

The Hoch-Marchaim 2003 publication (authored by the inventor) and cited by the Examiner, is a continuation of the research of 1998 and only adds that p14 is indeed the leader peptide of the MMTV env precursor; that the epitope recognized by M-66 contains a putative nuclear localization signal; that actinomycin D induced redistribution of p14/21 from the nucleolus to the nucleoplasm; and that p14 coimmunoprecipitated and colocalized with the cellular protein B23. Thus, Hoch-Marchaim 2003 does not add any information to the finding of Hoch-Marchaim 1998 that could be regarded as teaching, when combined with Pogo and Melana-I or Melana-II, the subject matter as claimed in the present application.

However, Applicant submits that the above cited reference, taken together with the findings described in Hoch-Marchaim 1998 would have led a person skilled in the art to understand that ***in the absence of p14 in acceptable cancer cell models, p14 cannot be used as a marker for determining the probability of a subject of having breast cancer***. Therefore, in contrast to the Examiner's assertion Applicant submits that a person of ordinary skill in the art would not have a reasonable expectation of success of achieving the present subject matter in view of the cited references.

In view of the remarks set forth herein, it is submitted that, whether taken alone or in combination, the cited art does not render the presently pending claims obvious within the meaning of 35 USC § 103 (a). Accordingly, the Examiner is respectfully requested to withdraw this rejection.

CONCLUSION

In view of the foregoing, Applicant submits that the application is in condition for immediate allowance. Early notice to that effect is earnestly solicited. The Examiner is invited to contact the undersigned attorney if it is believed that such contact will expedite the prosecution of the application.

In the event this paper is not timely filed, Applicant petitions for an appropriate extension of time. Please charge any fee deficiency or credit any overpayment to Deposit Account No. 14-0112.

Respectfully submitted,

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ATTACHMENT A

Nucleolar Localization of Mouse Mammary Tumor Virus Proteins in T-Cell Lymphomas

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To characterize novel proteins expressed in lymphoma cells, monoclonal antibodies (MAbs) were raised against variant S49 mouse lymphoma cells. Immunoperoxidase analysis with a specific MAb, named M-66, revealed nuclear localization with prominent staining in the nucleoli of both tumorigenic (T-63) cells and nontumorigenic, immunogenic (T-25-Adh) cells. Weak signals were also observed in the cytoplasm and plasma membrane of both cells. Western blot analysis with M-66 antibody revealed a 14-kDa protein in nuclear extracts of both T-25-Adh and T-63 cells. An additional nuclear 21-kDa protein was evident only in T-63 cells. M-66 identified several clones from a T-25-Adh cDNA expression library. These clones demonstrated extensive homology (~95% identity throughout their length) to the mouse mammary tumor virus (MMTV) env and LTR regions. Extensive amino acid sequence homology (~90% identity) between the clones and the env protein was observed. M-66 identified the 14-kDa protein in another MMTV bearing T-cell lymphoma, EL-4. Immunoperoxidase analysis of EL-4 cells with M-66 also revealed prominent nucleolar staining. MMTV-negative cells and MMTV-positive cells of nonlymphocytic origin were devoid of both 14- and 21-kDa proteins. Moreover, an anti-MMTV gp52 (env) antibody precipitated the 21-kDa protein in T-63 cells. We thus suggest that MMTV bearing T-cell lymphomas express nucleolar proteins translated from the env region of MMTV. © 1998 Academic Press

INTRODUCTION

Several reports have demonstrated that different viral proteins were localized to the nuclei and nucleoli of infected cells. The function of these proteins in the nucleus is known for some proteins and still has to be explored for others (D'agostino *et al.*, 1997; Kubota *et al.*, 1996; Mears *et al.*, 1995; Schoborg and Clements, 1994). To our knowledge, nuclear localization of proteins translated from the env or LTR regions of MMTV has not been reported.

MMTV is a Type B retrovirus that causes mammary adenocarcinoma. The most documented association of MMTV with nonmammary tumors is that with T-cell lymphomas (Racevskis, 1990; Yanagawa *et al.*, 1993). In T-cell lymphomas, the MMTV mRNAs of the gag and env genes are translated to their respective precursors, but usually not processed into mature viral proteins; mature viral particles are not produced (Racevskis, 1990). In these cells the proteolytic cleavage which generates gp52 and gp36 from the env polyprotein precursor (Redmond and Dickson, 1983) does not occur often. Another characteristic common to MMTV expressing lymphomas is the acquisition of amplified MMTV proviruses containing deletions in their U3 region of the LTR (Racevskis, 1990). The LTR contains an open reading frame (orf) which encodes the superantigen (Choi *et al.*, 1991; Acha-Orbea and MacDonald, 1995).

S49 is a T-cell lymphoma of BALB/c origin, which also contains amplified MMTV proviruses with a 430-bp deletion in the U3 region of the LTR (Hsu *et al.*, 1988).

We have used a highly tumorigenic S49 cell line variant derived by continuous passages in syngeneic mice (Hochman *et al.*, 1981). Cells from the 25th and the 63th passages (designated T-25 and T-63, respectively) grew as single cells in suspension culture, and gave rise to progressive tumors in syngeneic hosts. From T-25 cells, substrate adhesive variants (named T-25-Adh) were previously selected. T-25-Adh cells revealed impaired tumorigenicity and increased immunogenicity in immunocompetent mice (Hochman *et al.*, 1981, 1984). T-25-Adh cells were subsequently used to raise a variety of monoclonal antibodies (MAbs) in syngeneic BALB/c mice in order to identify novel proteins that characterize these cells (Hochman *et al.*, 1990).

Here we report that a newly derived MAb (designated M-66) identified MMTV env-related proteins in the nuclei/nucleoli of S49 and EL4 T-cell lymphomas, which contain acquired MMTV proviruses (Hsu *et al.*, 1988).

RESULTS

Cellular localization of proteins recognized by M-66

Mab M-66 was generated against T-25-Adh cells as previously described (Hochman *et al.*, 1990). Indirect

Sequence data from this article have been deposited with the Genbank Data Library under Accession Nos. AF043688, AF043689, and AF043690.

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0042-6822/98 $25.00

immunoperoxidase analysis of permeabilized T-63 and T-25-Adh cells demonstrated that the antigens recognized by M-66 were localized to the nucleus and particularly in nuclear bodies resembling nucleoli (Figs. 1A, 1B). Weak signals were associated with the cytoplasm and plasma membrane. No apparent differences between T-63 and T-25-Adh cells were observed (Figs. 1A, 1B). Immunoperoxidase electron microscopy (IEM) revealed prominent localization of M-66 in the nucleoli (Figs. 1D, 1F).

Differential expression of proteins recognized by M-66 in S49 variants

M-66 recognized in Western blot a 14-kDa protein in nontumorigenic T-25-Adh cells, while in tumorigenic T-25 and T-63 cells it recognized two proteins: the 14-kDa protein and an additional 21-kDa protein, specific to these cells (Fig. 2A). Western blot analysis with M-66 was carried out on nuclear and cytoplasmic fractions from T-63 and T-25-Adh cells. The 21-kDa protein was expressed essentially in the nuclear fraction of T-63 cells while the 14-kDa protein was distributed between the nuclear and cytoplasmic fractions of these cells (Fig. 2B). A similar distribution of the 14-kDa protein occurred also in T-25-Adh cells (not shown).

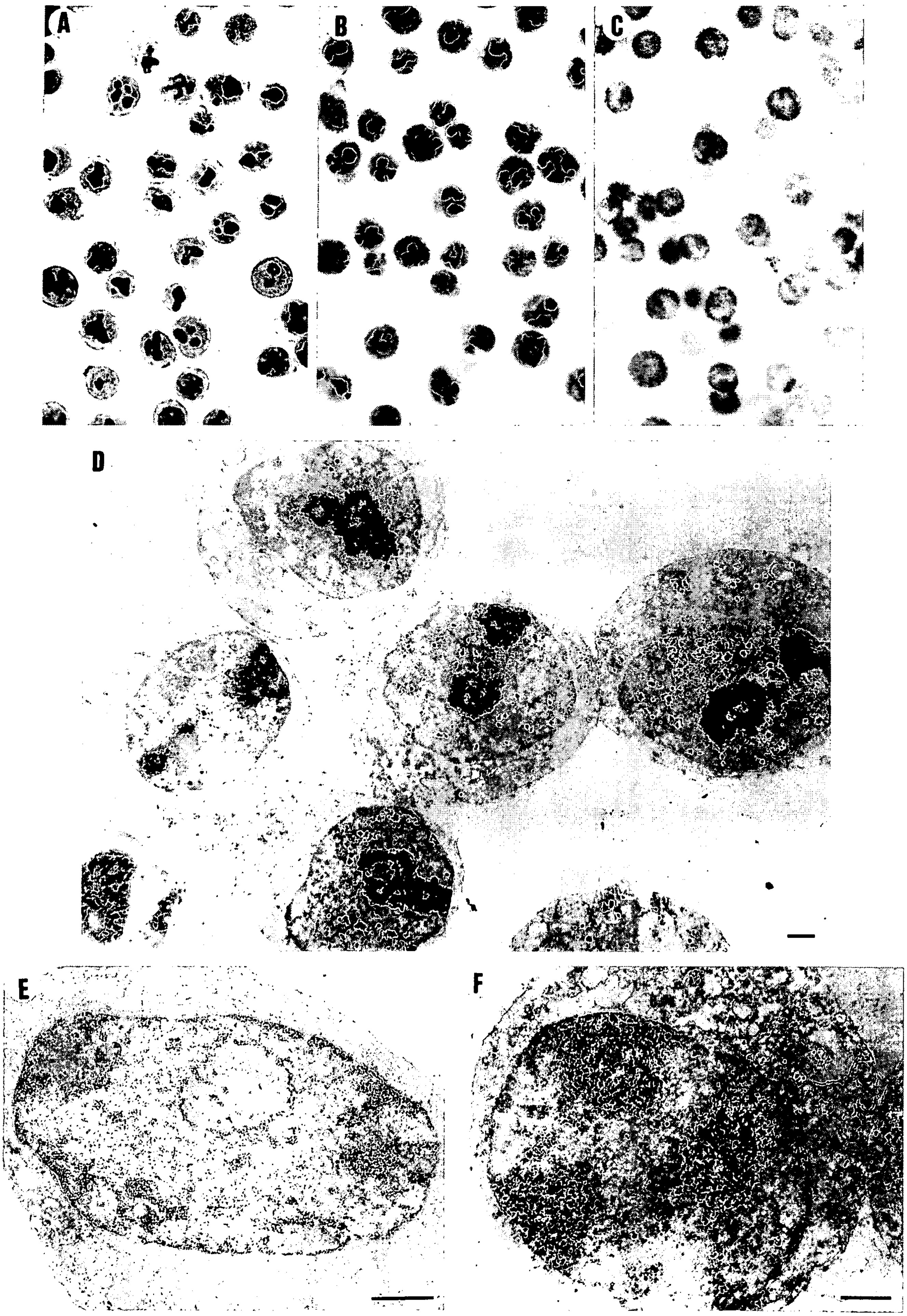
Screening of a T-25-ADH CDNA expression library with M-66

Since M-66 was raised against T-25-Adh cells, we used this MAb to screen a T-25-Adh cDNA expression library. This was carried out in order to identify the gene products recognized by this MAb. Out of ~250,000 clones screened, 7 positive clones (0.003%) were detected. Their plasmid DNA was purified and rescued with restriction enzymes (*Eco*RI and *Xho*I). Three different restriction patterns were observed among the 7 clones (not shown). The insert DNA from three clones, representing the three different restriction patterns, was used for sequence analysis. All of these clones, designated 66A (2.85 kb), 66B (2.77 kb), and 66C (1.79 kb), demonstrated extensive homology (~95% identity throughout their length) to the env and 3′LTR regions of MMTV (Fig. 3). These findings demonstrate that M-66 recognizes MMTV antigens. When compared to the published MMTV sequence from BR6 mice [(Moore *et al.*, 1987), GenBank Accession No. M15122], the 5′ ends of clones 66A and 66C were 44 and 88 bp upstream to the splice donor site of the env mRNA, respectively. The 5′ end of clone 66B was 1 bp upstream to the splice acceptor site of the env mRNA. The 3′ ends of clones 66A, 66B, and 66C were 113, 120, and 120 bp upstream to the MMTV 3′LTR endpoint, respectively. The sequence homology among these clones was ~98% identity and the differences in their length were due to internal deletions. All clones demonstrated a deletion in the 3′LTR region, common to amplified MMTV proviruses in T-cell lymphomas (Racevskis, 1990). Extensive amino acid sequence homology (~90% identity) between the different clones and the MMTV env region was found (Fig. 4). In clone 66A, this homology extended throughout the 688 amino acids of MMTV env polyprotein. Several point mutations were found in clone 66A, one of which generated a stop codon after 313 amino acids of the env polyprotein. In clone 66B, the amino acid sequence homology extended to the first 438 amino acids of the MMTV env polyprotein. This homology terminated in a deletion that overlapped with 36 amino acids from the C-terminus of gp52 and the first 4 amino acids of gp36. A translational frameshift was generated by this deletion. Amino acid sequence homology of 87% in the same translational reading frame of gp52 was found with the first 213 amino acids of MMTV superantigen (which is coded from the 3′LTR region). In clone 66C, the amino acid sequence homology extended to the first 260 amino acids of the env polyprotein. A 1-kb deletion terminated this homology. This deletion overlapped with the last 214 amino acids of gp52 and all but the last 40 amino acids from the C-terminus of gp36. The MMTV env precursor amino acid sequence (throughout the region homologous to the three cDNA clones) contains two clusters with a preponderance of basic amino acids (Fig. 4), which may potentially serve as nuclear localization sequences (NLSs).

Western blot analysis and immunoprecipitation of MMTV antigens

Western blot analysis of T-63 and T-25-Adh cell extracts was carried out using an anti-MMTV polyclonal antibody, R101 (a generous gift from I. Keydar, Tel-Aviv University). A faint band of a 21-kDa protein was evident in T-63 cells but not in T-25-Adh cells (Fig. 5A). The 14-kDa protein was not observed. To find out whether the 21-kDa protein was the protein recognized by M-66, we carried out an immunoprecipitation of T-63 cell extracts with R101 and subsequent Western blot with M-66. The 21-kDa protein (Fig. 5B) was observed and established the M-66 21-kDa band as a MMTV protein. Furthermore, immunoprecipitation of T-63 lysates with M-66 followed by Western blot with an anti MMTV (env) gp52 polyclonal antibody (a generous gift from G. Smith, National Institutes of Health-Bethesda, MD) demonstrated that the anti gp52 antibody also recognized the 21-kDa protein (but not the 14-kDa protein, Fig. 5C). A reciprocal analysis (immunoprecipitation with anti gp52 and subsequent Western blot with M-66) gave the same result (not shown).

The immunoprecipitation studies verified that the nuclear 21-kDa protein in T-63 cells is MMTV env-related. Based on the T-25-Adh cDNA library screening, we assume that the 14-kDa protein (the sole protein recognized by M-66 in these cells) is also MMTV env-related,


A
B
C
D
E
F

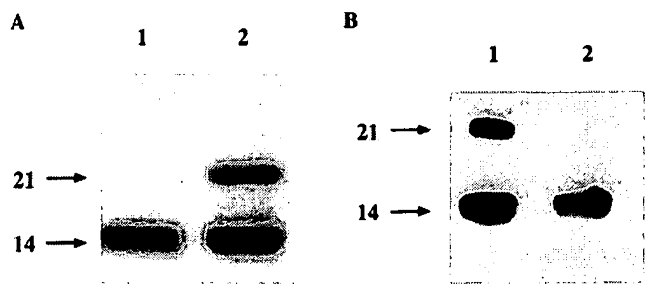


FIG. 2. Western blot analysis of T-63 and T-25-Adh cells with M-66. Arrows indicate molecular masses in kDa. (A) T-25-Adh (40 μg protein, lane 1) and T-63 (48 μg protein, lane 2) lysates; (B) 35 μg protein of nuclear (lane 1) and cytoplasmic (lane 2) lysates (see Materials and Methods) from T-63 cells.

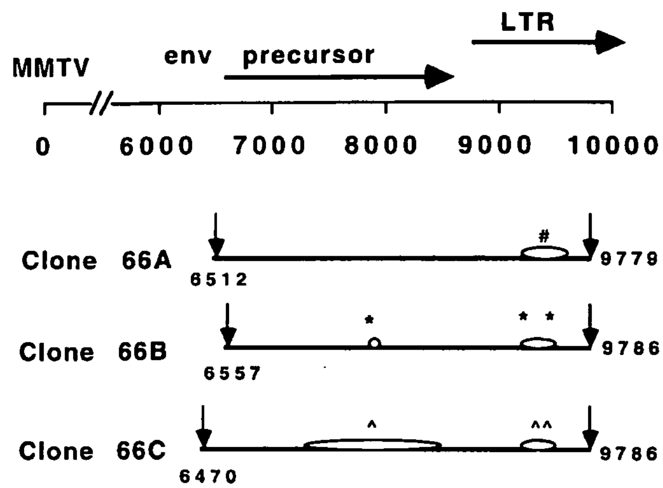


FIG. 3. cDNA clones identified by M-66. Schematic diagram of the clones as compared to the published MMTV sequence from BR6 mice [(Moore *et al.*, 1987), GenBank Accession No. M15122]. Arrows (accompanied by numbers) represent the start and the end points of the three clones. The ATG codon of the MMTV env polyprotein starts at nucleotide 6559. Deletions in the env and LTR regions of the different clones are indicated: (#) Deletion 9211–9657. (*) Deletion 7872–7989. (**) Deletion 9204–9557. (^) Deletion 7343–8499. (^^) Deletion 9202–9554.

and that both proteins are produced through differential processing of the MMTV env precursor. One may speculate that the 21- and 14-kDa proteins share the 14-kDa component recognized by M-66. The additional 7-kDa element in the 21-kDa protein may contain the epitopes recognized by anti-MMTV and anti-gp52 antibodies.

In vitro transcription–translation

The cDNA clones 66A, 66B, and 66C were subjected to *in vitro* coupled transcription–translation with reticulocyte lysate (see Materials and Methods) and separated by SDS–PAGE. The molecular weights of the major bands, representing the proteins encoded from clones 66A, 66B, and 66C, were 35, 50, and 34 kDa, respectively (Fig. 6A). This is expected from translation starting at the first ATG codon and terminating at the first stop codon of each clone.

The proteins translated *in vitro* from the three cDNA clones were subjected to immunoprecipitation with M-66 and SDS–PAGE. The major bands that were visualized following immunoprecipitation (Fig. 6B) corresponded to the major proteins synthesized (Fig. 6A). The signal from the immunoprecipitation of clone 66C product was very faint (Fig. 6B). This may be due to lower affinity of M-66 antibody to the translation product of this clone.

These findings strengthen our assumption that the proteins recognized by M-66 in the lymphoma cell lysates (i.e., the 14- and 21-kDa proteins) are produced (from a precursor(s) of higher molecular weight) through posttranslational processing. Furthermore, the immunoprecipitation results establish once more that M-66 recognized MMTV env-related antigens.

Analysis of other MMTV-positive and -negative cells

Upon immunoperoxidase staining with M-66, EL4 cells [MMTV-positive T-cell lymphoma from C57BL/6 mice (Racevskis, 1990)]—kindly provided by B. Leshem, The Hebrew University of Jerusalem—demonstrated a nucleolar pattern similar to T-63 and T-25-Adh cells (Fig. 7). EL4 cells also revealed the 14-kDa protein in Western blot analysis with M-66 (Fig. 7, inset). The following cells were devoid (upon Western blot with M-66) of either the 14- or the 21-kDa protein: (1) MMTV-negative PIR-2 (Yefenof *et al.*, 1982)—kindly provided by E. Yefenof, the Hebrew University of Jerusalem, and 136.5 murine T-cell lymphomas, (Haas, 1974). (2) MMTV-positive mammary carcinoma cell line Mm5MT (Owens and Hacket, 1972)—kindly provided by I. Keydar, Tel-Aviv University. (3) Two independent CZECH 2 MMTV-positive mammary tumor tissues—a generous gift from R. Callahan, National Institutes of Health (Bethesda, MD). (4) Syngeneic Balb/C spleen and liver cells. Taken together with the above mentioned findings, it is conceivable that the 14-kDa band is also a MMTV protein, and it is suggested that M-66 identified MMTV nuclear/nucleolar antigens specific to MMTV bearing T-cell lymphomas.

FIG. 1. Cellular localization of proteins identified by M-66 in S49 cells. (A–C) Immunoperoxidase staining of (A) T-63 cells, (B) T-25-Adh cells, (C) negative control (blocking buffer instead of M-66) of T-25-Adh cells. Original magnification: ×1000. (D, F) Immunoperoxidase electron photomicrograph of ultrathin sections of T-25-Adh cells following reaction with M-66. Bar, 1 μm. (D) Original magnification: ×5500. (F) Original magnification: ×10,000. (E) Electron photomicrograph of control section devoid of the first (M-66) antibody. Section was also stained with uranyl acetate and lead citrate to visualize the boundaries of the nucleus and nucleoli. No peroxidase reaction is evident. Original magnification: ×12,500.

```
     1                                                    50
mmtv ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^M PKHQSGSPTD SSDLLLSGKK
66A  ^^^^^^^^^^ ^^^^TDSPQG TAVSPTEKR- -N-------G ----------
66B  ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^- -N-------G ----------
66C  CFVSILSGYH HKSGTDSPQG TAVSPTEKR- -N-------G ----------

     51                                                  100
mmtv QRPHLALRRK RRREMRKINR KVPRMNLVPI KEKTAWQHLQ ALISEAEEVL
66A  ---------- ---------- --R----DI- ---------- ----------
66B  ---------- -----K---- --R----DI- ---------- ----------
66C  ---------- ---------- --R----DI- ---------- ----------

     101                                                 150
mmtv KTSQTPQTSL TLFLALLSVL GPPPVTGESY WAYLPKPPIL HPVGWGSTDP
66A  ---------- ---------- ---------- ---------- -----E----
66B  ---------- ---------- ---------- ---------- ----------
66C  ---------- ---------- ---------- ---------- ----------

     151                                                 200
mmtv IRVLTNQTMY LGGSPDFHGF RNMSGNVHFE GKSDTLPICL SFSFSTPTGC
66A  ---------- ---------- ---------- ---------F ----------
66B  ---------- ---------- ---------- ---------F ----------
66C  ---------- ---------- ---------- ---------F ----------

     201                                                 250
mmtv FQVDKQVFLS DTPTVDNNKP GGKGDKRRMW ELWLTTLGNS GANTKLVPIK
66A  ---------- ---------- ---------- K--------- -----R----
66B  ---------- ---------- ---------- ---------- -----R----
66C  ---------- ---------- ---------- ---------- -----R----

     251                                                 300
mmtv KKLPPKYPHC QIAFKKDAFW EGDESAPPRW LPCAFPDQGV SFSPKGALGL
66A  ---------- ---------- ---------- ---------- ----------
66B  ---------- ---------- ---------- ---------- ----------
66C  ---------- ---------- ---------- ---------C FAKSLDQVQS

     301                                                 350
mmtv LWDFSLPSPS VDQSDQIKSK KNLFGNYTPP VNKEVHRWYE AGWVEPTWFW
66A  ---------N IN--G---N- -D-Y------ ---------- --*-------
66B  ---------N I---G---N- -D-YE----- ---------- ----------
66C  DLNVL-LKKK KGGNAAPAAE MVELPRVSYT *RRSS-GVVS HPGRPICAQT
```

FIG. 4. Amino acid sequence of clones 66A, 66B, and 66C compared to MMTV. Numbering of the bases begins at the first base of clone 66C. The sequence is drawn up to 60 bp after the first deletion in env region of clone 66C (the first deletion among the three clones). The beginning of this deletion is marked by a dashed arrow. The MMTV sequence was obtained from BR6 mice (GenBank Accession No. M15122). The MMTV sequence is drawn from the first methionine of the env polyprotein precursor, which is marked by an arrow. Nucleotides identical to the MMTV sequence from BR6 mice are represented by dashes, whereas altered bases are indicated by letters. Shaded boxes indicate amino acid substitutions which are common to all three clones and differ from both the MMTV sequence from BR6 mice (GenBank Accession No. M15122) and the MMTV sequence from GR mice [(Redmond and Dickson, 1983), GenBank Accession No. K01788, not shown)]. Clusters with preponderance of basic amino acids are underlined. Asterisks indicate stop codons.

DISCUSSION

A monoclonal antibody (M-66) raised against nontumorigenic, immunogenic T-25-Adh cells recognized one protein (14 kDa) in the nucleoli of these cells, and two proteins (14 and 21 kDa) in the nucleoli of tumorigenic T-63 cells. Since the 21-kDa protein was immunoprecipitated by both anti-MMTV and anti-gp52 (env) antibodies, it is suggested that this protein, localized to the nucleoli of T-63 cells, is MMTV env-related.

With regard to the 14-kDa protein, we cannot rule out the possibility that it may not be MMTV-related. However, several lines of evidence suggest that this protein is indeed also a MMTV protein, probably env-related: (1) M-66 identified only MMTV clones from a T-25-Adh cDNA expression library, with an extensive amino acid sequence homolgy to the MMTV env polyprotein. (2) The 14-kDa protein is the sole protein recognized by M-66 in T-25-Adh cell lysates. (3) M-66 identified the 14-kDa protein only in the nuclei of MMTV bearing T-cell lymphomas (S49 and EL4) but not in MMTV-negative T-cell lymphomas (PIR-2 and 136.5).

Both env and LTR regions of the cDNA clones demonstrated deletions and amino acid substitutions. The deletion in the U3 LTR region of all clones indicates that they were derived from the acquired MMTV proviruses common to T-cell lymphomas. Racevskis (1990) has demonstrated the production of deletions and premature termination of env mRNAs in two T-cell lymphoma lines (including EL4), but did not find any smaller env transcript in S49 cells (Racevskis, 1990). A 1-kb deletion is

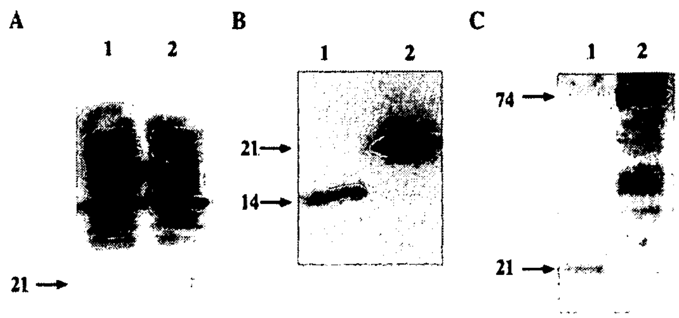


FIG. 5. Western blot analysis of T-63 and T-25-Adh cells with R101 (anti-MMTV polyclonal antibody), M-66, and anti-MMTV gp52. Arrows indicate molecular masses in kDa. (A) T-25-Adh cell extract (lane 1) and T-63 cell extract (lane 2) were subjected to Western blot with anti-MMTV (R101) antibody and an HRP-linked anti-rabbit Ig. (B) T-63 cell lysate was immunoprecipitated with R101; 48 μg protein of the immunoprecipitation supernatant (lane 1) and pellet (lane 2) was subjected to Western blot with M-66 and a HRP-linked anti-mouse Ig. (C) T-63 cell lysate was immunoprecipitated with M-66; 24 μg protein of the immunoprecipitation pellet (lane 1) and supernatant (lane 2) was subjected to Western blot with anti-MMTV gp52 and an HRP-linked anti-rabbit Ig.

evident in the env region of clone 66C (Fig. 3). Whether this deletion is specific to T-25-Adh cells (and may play a role in their biological characteristics) or whether it is expressed in other S49 cell variants is presently under investigation. In this context it is noteworthy that we have recently established a role for MMTV in rendering T-25-Adh cells immunologically foreign to syngeneic hosts (Mador *et al.*, 1997). This was based on differing morphogenesis of cytoplasmic MMTV precursors, between T-25-Adh and parental, tumorigenic T-25 cells, following their *in vivo* inoculation. The present work demonstrates another difference between T-25 and T-25-Adh cells that relates to MMTV. At this stage it is intriguing to speculate on a connection between the previous and present findings on MMTV in regulating the xenogenization of S49 cells.

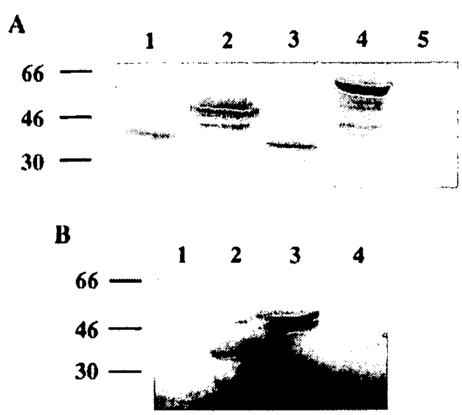


FIG. 6. *In vitro* transcription–translation and immunoprecipitation of MMTV cDNA clones. (A) Transcription–translation products of clones 66A (lane 1), 66B (lane 2), 66C (lane 3), luciferase control DNA (lane 4), and no DNA control (lane 5). (B) Immunoprecipitation of the transcription–translation products of luciferase control DNA (lane 1) and cDNA clones 66A (lane 2), 66B (lane 3), and 66C (lane 4) by M-66 (see Materials and Methods). Molecular masses are shown in kDa.

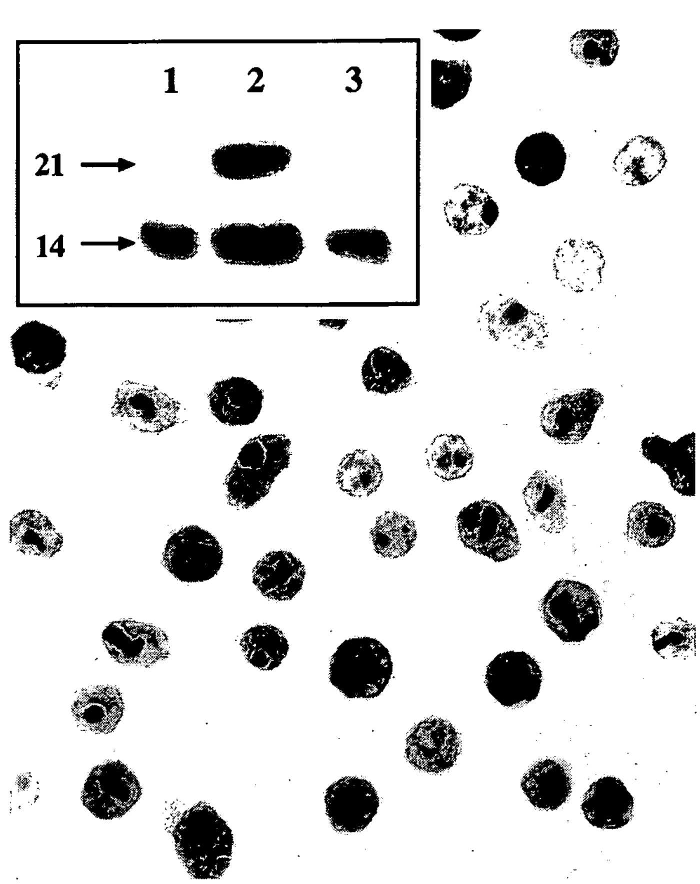


FIG. 7. Immunoperoxidase analysis of EL4 cells with M-66. Inset, Western blot of T-25-Adh (lane 1), T-63 (lane 2), and EL4 (lane 3) cells with M-66.

Figure 4 demonstrates that there are three amino acid substitutions in the env region which are common to the three cDNA clones and differ from the published sequence of MMTV from BR6 mice (Moore *et al.*, 1987) and from GR mice (Redmond and Dickson, 1983). We assume that the recognition epitope of M-66 may include at least one of these substitutions. This may explain why M-66 did not recognize the 74-kDa band of the MMTV env polyprotein precursor, which can be seen in Western blot with anti gp52 (Fig. 5C, lane 2). There are three BALB/c MMTV endogenous proviruses (Cho *et al.*, 1995). We suggest that except for the endogenous MMTV env precursor, which generates the 74-kDa protein in S49 cells, the acquired MMTV proviruses in MMTV bearing T-cell lymphomas, represented in T-25-Adh cells by the three cDNA clones, give rise to aberrant MMTV env precursors. These may undergo rapid processing to generate the 14- and 21-kDa proteins. This suggestion is also supported by the transcription–translation–immunoprecipitation analysis (see above).

Certain proteins are transported to the nucleus if they contain an active nuclear localization sequence (NLS) (Silver, 1991; Laskey *et al.*, 1996). NLSs are usually short (generally less than 12 amino acids) and contain a high proportion of positively charged amino acids. Many nuclear proteins contain a bipartite motif which was defined as two basic residues, a spacer of any 10 amino

acids, followed by a second basic cluster in which at least 3 out of 5 amino acids are basic (Dingwall and Laskey, 1991). Differences in spacer length were also found (Dingwall and Laskey, 1991). The MMTV env precursor amino acid sequence (throughout the region homologous in the three cDNA clones) contains two clusters with a preponderance of basic amino acids (Fig. 4). The first of these (amino acids 29–45 from the first methionine of the env precursor, Fig. 4) contains nine arginines, which may serve as candidates for nucleolar targeting (D'agostino *et al.*, 1997). At present we may only speculate that one of these clusters in the 14- and the 21-kDa proteins (but not in the env precursor), is exposed to the NLS receptor, importin (Laskey *et al.*, 1996) and thus can serve as NLS in the two former proteins. Another possibility is that an amino acid substitution, which occurs in the proteins recognized by M-66 (for instance, the change from leucine to arginine in position 217 at the basic amino acid cluster starting at position 216 of the env precursor, Fig. 4) may be instrumental for the nuclear import of these proteins.

Previous experiments demonstrated that small proteins can enter the nucleus without using NLS (Silver, 1991). Therefore, the possibility that the entry of the 14- and 21-kDa proteins into the nucleus is independent of NLS may also be valid.

The function of the MMTV proteins in the nucleoli is presently unknown. The nucleolus is the site of preribosome formation. Other studies suggested its role in poly(A)$^+$ RNA transport and processing and in viral replication (Li, 1997). Several retroviral proteins are localized to the nucleoli of infected cells. For example, two nucleolar/nuclear proteins encoded by the human T-cell lymphotropic virus type 1 (HTLV-1) are Rex and Tof. While Rex is a posttranscriptional regulator that shuttles between the nucleus and the cytoplasm, Tof is a protein of unknown function which appears to remain stably associated with nuclear/nucleolar components (D'agostino *et al.*, 1997). A recent study suggests that Tof may modulate RNA expression at a level distinct from that of Rex at a later phase of the viral life cycle (D'agostino *et al.*, 1997). The nucleolar/nuclear localization signal of Tof contains two arginine-rich segments, the first of which was found to be necessary for nucleolar but not for nuclear targeting (D'agostino *et al.*, 1997).

In analogy to other retroviral proteins localized to the nuclei and nucleoli of infected cells, it is of interest to study wether the 14- and the 21-kDa proteins play a role in transcriptional or posttranscriptional regulation of the MMTV RNA.

The induction of T-cell lymphomas by MMTV has been attributed to the loss of a negative regulatory element in the LTR due to deletions and rearrangements (Hsu *et al.*, 1988; Yanagawa *et al.*, 1993). A transcriptional activator in the MMTV env gene, specific to T cells, was also described. It has been speculated that this transcriptional activator may also participate in the induction of T-cell lymphomas (Miller *et al.*, 1992; Sambasivarao and Paetkau, 1996).

Based on our findings, it is further tempting to speculate that the nuclear/nucleolar localization of MMTV proteins in T-cell lymphomas may be involved in the induction of these lymphomas (through the 14-kDa protein) or may contribute some selective advantage to their tumorigenicity (through the 21-kDa protein).

MATERIALS AND METHODS

Cells

Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with either 10% heat-inactivated horse serum (S49 variant cells, PIR-2 and 136.5 lymphomas) or 10% heat-inactivated fetal bovine serum (Mm5MT and EL4 cells).

Monoclonal antibody M-66

MAb M-66 (IgG2b) was generated against T-25-Adh cells in syngeneic BALB/c mice as previously described (Hochman *et al.*, 1990). During initial immunofluorescence screening studies on intact T-25-Adh cells, M-66 reacted faintly with the surface of these cells.

Immunoperoxidase analysis

Cells were washed with 0.1 M sodium cacodilate buffer (SC), pH 7, and fixed with 2% paraformaldehyde in SC. The cells were then washed again in SC. Drops of cells were mounted on gelatin-subbed slides and dried. Cells were incubated in 0.5% borohydride in PBS for 20 min and washed. Blocking was carried out using 2% bovine serum albumin and 0.5% glycine in phosphate-buffered saline (PBS) for 1 h. Triton X-100, 0.4%, was included only for light microscopy. The cells were first incubated with M-66 overnight at 4°C, washed, and then incubated for 2 h with anti-mouse IgG-biotinylated secondary antibody. Avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit) was added for additional 2 h. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride (DAB) with H_2O_2. For light microscopy slides were dehydrated, cleared, and coverslipped with Entellan (Merck). For electron microscopy, cells were postfixed in osmium tetroxide, dehydrated, infiltrated with resin (EMbed-812, electron microscopy sciences), and embedded in Epon EMbed-812. Ultrathin sections were collected on nickel grids and visualized in a Philips 300 electron microscope.

Western blot analysis

Cells were lysed by SDS–PAGE sample buffer, separated by 15% SDS–PAGE (Laemli, 1970), and transferred electrically to nitrocellulose. After blocking for 1 h with 5% nonfat dry milk in PBS containing Tween 20 (0.1%

vol/vol), the nitrocellulose was incubated for 1 h with M-66, rabbit anti-MMTV polyclonal antibody R101, or goat anti-MMTV gp52. Following washing with PBS–Tween-20, the membrane was incubated for 1 h with anti-mouse, anti-rabbit, or anti-goat Ig, horseradish peroxidase (HRP)-linked $F(ab')_2$ fragment (Amersham), respectively. The nitrocellulose was washed again and ECL substrate was used for detection (Amersham).

Immunoprecipitation

Immunoprecipitation was carried out as previously described (Aroeti *et al.*, 1993). Briefly, cells were lysed with 0.5% SDS lysis buffer and the lysates were precleared with protein A–Sepharose CL-4B (Pharmacia) and incubated with M-66, R101, or anti-gp52 antibodies. The appropriate proteins were precipitated after an additional incubation with protein A–sepharose and centrifugation. The pellet was washed and SDS–PAGE sample buffer was added. After boiling and centrifugation the proteins were analyzed by Western blot.

Nuclear and cytoplasmic fractions

Pure nuclei were prepared as previously described (Sperling *et al.*, 1985), except that the lysed cells were loaded on a 25% glycerol cushion and centrifuged at 750*g* for 5 min at 4°C. The postnuclear supernatant was designated as cytoplasmic fraction. The nuclear pellet was washed and lysed by SDS–PAGE sample buffer.

Screening of a cDNA expression library

A T-25-Adh cDNA expression library (in Uni-ZAP XR vector, from ZAP-cDNA synthesis kit, Stratagene) was screened according to the manufacturer's instruction manual for the picoBlue immunoscreening kit (Stratagene). Briefly, the fusion proteins were expressed in Uni-ZAP XR vector and transfered to nitrocellulose (Schleicher & Schuell). The nitrocellulose sheets were incubated with M-66 for 1 h. After washing, the membranes were incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) were used for detection. Positive clones were picked for secondary and tertiary screenings. The pBluescript phagemid was excised from positive clones and the DNA was purified and used for further analysis.

DNA methodology and reagents

Restriction enzyme digestion and agarose gel electrophoresis were carried out by standard protocols (Sambrook *et al.*, 1989). DNA sequencing was carried out using the dye terminator cycle sequencing and analyzed on an ABI PRISM 377 DNA sequencer (Perkin Elmer) and sequence analysis software. Oligonucleotides were obtained from the Unit of Development of Molecular Biology and Genetic Engineering (Hadassah University Hospital, Jerusalem, Israel). Restriction enzymes were obtained from Promega. DNA purification was carried out using the Qiagen plasmid midi kit. The Wisconsin package, version 9.0, genetic computer group (GCG, Madison, WI), was applied.

In vitro transcription–translation and immunoprecipitation

cDNA clones (1 μg plasmid DNA) were subjected to *in vitro* transcription–translation using the TNT T3 coupled reticulocyte lysate kit (Promega), according to the manufacturer's instructions. Redivue L-[^{35}S]methionine (Amersham) was used for protein labeling; 5 μl from each reaction mixture (10%) was combined with 20 μl of SDS sample buffer, and 10 μl from this mixture was subjected to 15% SDS–PAGE. Immunoprecipitation of the transcription–translation products with M-66 was carried out as described above, except that the immunoprecipitated proteins were separated on 15% SDS–PAGE and visualized using autoradiography of the dried gel.

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